SELDI-TOF MS Profiling of Plasma Proteins in Ovarian Cancer

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SUMMARY

Objective: Proteomic profiling of plasma or serum is a technique to identify new biomarkers in disease. The objective of this study was to identify new plasma biomarkers in ovarian cancer patients using mass spectrometry protein profiling and artificial intelligence.

Methods: A total of 65 plasma samples obtained from women with ovarian cancer (n = 35) and age-matched disease-free controls (n = 30) were applied to anion exchange protein chips for protein profiling by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS).

Results: SELDI-TOF MS was highly reproducible in detecting ovarian tumor-specific protein profiles. One protein peak (relative molecular mass, Mr, 11,537 Da) was identified in plasma from women with ovarian cancer but not in controls. Two peaks, Mr 5,147 and 8,780 Da, were present in the plasma of controls but not of women with ovarian cancer. After a training analysis, classification analysis generated by univariant or linear combination split was performed to reach a discriminant protein signature pattern. After cross validation, a sensitivity of 84% and specificity of 89% for all studied cases and controls was reached.

Conclusion: This study clearly demonstrates that the combined technology of SELDI-TOF MS and artificial intelligence is effective in distinguishing protein expression between normal and ovarian cancer plasma. The identified protein peaks may be candidate proteins for early detection of ovarian cancer or evaluation of therapeutic response. [*Taiwanese J Obstet Gynecol* 2006;45(1):26–32]

Key Words: protein chip, ovarian cancer, SELDI-TOF mass spectrometry

Introduction

Of the gynecologic malignancies, ovarian cancer has the highest mortality rate. It often eludes the clinician because of the lack of early symptoms and signs, presenting at a late clinical stage in more than 80% of patients; ovarian cancer is associated with a low 5-year survival of 35% in this population [1–5]. In contrast, the 5-year survival for patients with stage I ovarian cancer exceeds 90% and most patients are cured by surgery alone [3–5]. Therefore, increasing the number of women diagnosed with stage I disease should have a direct effect on the mortality and economics of this cancer.

The identification of cancer biomarkers provides the possibility for early detection, better monitoring of tumor progression, and even targeting therapy [1–7]. Traditional strategies for cancer biomarker identification used tumor cells to immunize animals and screen for antibodies that could efficiently recognize the antigen [8]. This approach is limited by its high cost and labor intensity but has produced the best-known marker approved for ovarian cancer monitoring, CA125 [8]. More recently, tumor mRNA has been compared with normal tissue mRNA to identify upregulated genes in cancer tissue, using cDNA microarrays to identify a

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variety of markers, including prostasin, osteopontin, and He4 [9–11]. A limitation of the cDNA microarray approach is that transcriptional differences in the tumor do not completely reflect the protein observed peripherally, because many protein–protein interactions and post-translational modifications may change the protein patterns found in blood.

Recently, a protein chip coupled with surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) has been developed to facilitate protein profiling of complex biological mixtures, with high efficacy of discovery of cancer protein markers in serum or plasma [12–14]. The objective of this study was to determine whether SELDI-TOF MS profiling of plasma proteins coupled with an artificial intelligence data analysis algorithm could effectively discriminate between normal controls and patients with malignant ovarian cancer. Using a standardized training set, we demonstrated that our SELDI protein profiling approach could accurately discriminate between plasma from patients with ovarian cancer and that from women who do not have ovarian disease. Our results form the basis for initiating further evaluation and validation to assess the potential of this SELDI proteomic classification system for the early detection and diagnosis of ovarian cancer, a clinically important lethal cancer.

Methods

Patients and specimens

All patient-related biological specimens were collected and archived under protocols approved by the institutional review board of the Tri-Service General Hospital, Taiwan, as described previously [15]. Informed consent was obtained from each patient and control subject. Plasma was collected preoperatively from women requiring surgery for ovarian tumors. Healthy, age-matched (< 3 years) controls were enrolled from women attending the clinic for routine Pap screening. The current analysis was based on plasma specimens from 30 patients who had surgically and histologically proven ovarian cancers (15 serous cystadenocarcinoma, 5 mucinous cystadenocarcinoma, 2 endometrioid adenocarcinoma, 2 malignant Brenner tumors, 3 poorly differentiated adenocarcinoma, and 3 secondary metastatic tumors). There were 35 plasma specimens from age-matched controls, who had no history of gynecologic tumors and had a normal pelvic examination and/or pelvic sonography. Plasma was prepared from EGTA-anticoagulated peripheral blood, aliquoted, and stored at –80° C, generally within 4 hours after collection.

Materials

Strong anion exchange (SAX) protein chips and other SELDI-related materials were obtained from Ciphergen Biosystems (Fremont, CA, USA). NP-40 and phosphate-buffered saline (PBS) came from Invitrogen (Carlsbad, CA, USA) and trifluoroacetic acid was from Pierce (Rockford, IL, USA). All other general chemicals were purchased from Sigma (St. Louis, MO, USA).

Matrix preparation and SELDI calibration

Calibration of the SELDI system was carried out before each analytical session. Sinapinic acid matrix was prepared freshly on the day of use by sequential addition of 125 μL of acetonitrile and 125 μL of 1% trifluoroacetic acid to an aliquot of matrix. This was vortexed and stored in the dark. Before use, the matrix was microfuged at 13,000 rpm for 1 minute. For calibration purposes, an H4 chip spot was loaded with 2 μL of matrix containing all-in-one protein standard. The mix included cytochrome C (12.2 kDa), myoglobin (17.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (35.7 kDa), albumin (66.4 kDa), and β-galactosidase (116.4 kDa). After air drying, the mixture was analyzed on the SELDI system using high mass acquisition of 40 kDa, optimum mass range of 3–20 kDa, laser intensity of 210, sensitivity of 10, and 50 collecting transients across the spot surface. Calibration was performed using single- and double-charged peaks for each calibrant.

SAX2 protein chip analysis

Diluted plasma samples (1:10) were processed on SAX2 chips according to the manufacturer's protocols (Ciphergen Biosystems). Briefly, the array spots were preactivated with binding buffer (1 μL PBS/0.1% Triton X-100, pH 7.5) at room temperature for 15 minutes in a humidifying chamber. Each diluted sample (3 μL) was spotted onto preactivated SAX2 chips and incubated in a humidity chamber for 30 minutes at room temperature. The chips were washed twice with binding buffer and once with high-performance liquid chromatography-grade water, and then air-dried. The chips were then sequentially treated with saturated sinapinic acid (with 50% acetonitrile and 0.5% trifluoroacetic acid) and analyzed with the Ciphergen ProteinChip Reader, PBSII (Ciphergen Biosystems).

SELDI data analysis

Spectra were analyzed with Ciphergen ProteinChip software (version 3.1) and normalized using total ion current. Peak clustering in the 2–30-kDa range was performed using Biomarker Wizard Software (Ciphergen Biosystems) at settings that provide a 5% minimum peak threshold, 0.2% mass window, and 2–3% signal/

noise determination. Intensity values for each peak were then averaged for each duplicate sample pair analyzed and input into BioMarker Patterns software (Ciphergen Biosystems) for classification tree analysis as described in the instruction manual.

In classification trees established by univariate split selection, the data are split into two nodes, using one rule at a time in the form of a question. The splitting decisions in this case were based on the normalized intensity levels of peaks from the SELDI protein expression profile. Each peak or cluster identified from the SELDI profile is therefore a variable in the classification process. The process of splitting is continued until terminal nodes are reached and further splitting has no gain in data classification. Multiple classification trees were generated using this process, and the best-performing tree was chosen for testing. During the analysis, a pruning step occurs in which branches are removed and the cost of the removal is determined to establish a minimal tree size. This is referred to as a "learning set". The decision tree was then subjected to cross validation. In this step, the data are partitioned such that randomly selected samples are categorized to ensure that the decision tree is valid. Cross validation is used if data are insufficient for a separate test sample. In such cases, Biomarker grows a maximal tree on the entire learning sample. This is the tree that will be pruned. Biomarker then proceeds by dividing the learning sample into 10 roughly equal parts, each containing a similar distribution for the dependent variable. Biomarker takes the first nine parts of the data, constructs the largest possible tree, and uses the remaining tenth of the data to obtain initial estimates of the error rate of selected sub-trees. The same process is then repeated (growing the largest possible tree) on another 9/10 of the data while using a different tenth part as the test sample. The process continues until each part of the data has been held in reserve one time as a test sample. The results of the 10 mini-test samples are then combined to form error rates for trees of each possible size; these error rates are applied to the tree based on the entire learning sample. The upshot of this complex process is a set of fairly reliable estimates of the independent predictive accuracy of the tree. This allows determination of how well any tree will perform on completely fresh data—even if there is no independent test sample. Because the conventional methods of assessing tree accuracy can be wildly optimistic, cross validation is the method Biomarker normally uses to obtain objective measures for smaller data sets.

The most common way to run Biomarker is to let Biomarker try to split each node by looking at one variable at a time. The second split selection method is the linear combination split option for ordered predictor variables. An attempt was made to create another decision tree by comparing several variables at a time using linear combination splitting. To illustrate this point, some artificial data were created using the equation:

$$\text{linear combination split } Y = AX1 + BX2 + CX3$$

where $X1$, $X2$, and $X3$ are the predictor variables and A, B, and C are the corresponding linear discriminant function coefficients. A score for each patient on the linear discriminant function would be computed as a composite of each patient's measurements on the three predictor variables, weighted by the respective discriminant function coefficients. The predicted classification of each patient as control or diseased would be made by simultaneously considering the patient's scores on the three predictor variables.

Statistical analysis

Specificity was calculated as the ratio of the number of negative samples correctly classified to the total number of true negative samples. Sensitivity was calculated as the ratio of the number of correctly classified diseased samples to the total number of diseased samples. Comparison of relative peak intensity levels between groups was calculated using Student's *t* test.

Results

SELDI protein spectra

The reproducibility of SELDI spectra, i.e. mass location and intensity between chips, was determined using the pooled normal plasma quality control sample. Five protein peaks in the range of 2,000–30,000 Da were randomly selected over the course of the study and were used to calculate the coefficient of variance. The coefficients of variance for peak location and normalized intensity were 0.05% and 15%, respectively. Plasma spectra from patients and control women do not show large variations (Figure 1). Therefore, small variations between different sample groups could be used for biomarker discovery. SELDI-TOF mass spectra of plasma samples were generated. Figure 1 shows that a major peak with *Mr* of 8,780.48 Da was consistently lost in plasma of women with ovarian cancers and a peak with *Mr* of 11,537.7 Da was consistently gained in such plasma.

Establishment of a diagnostic decision tree

Peak labeling was performed with the Biomarker

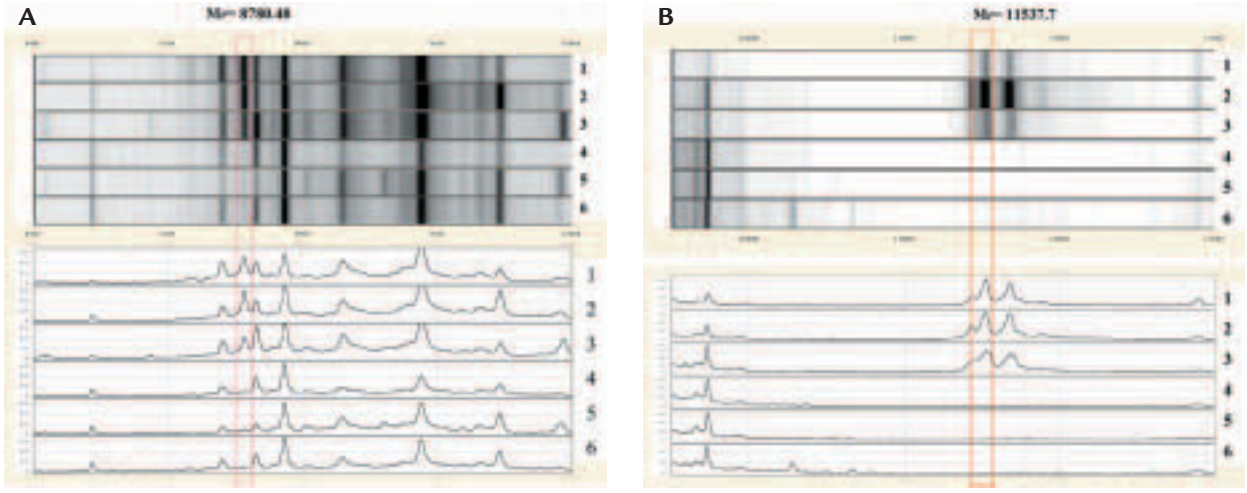

Figure 1. Representative differential surface-enhanced laser desorption/ionization time-of-flight mass spectra of ovarian cancer plasma. Gel (upper panel) and spectral (lower panel) view of plasma protein spectra in low (A) and high (B) molecular weight range of representative cases, where spectra 1, 2, and 3 were from control women and spectra 4, 5, and 6 were from ovarian cancer patients. In (A), a protein peak of Mr 8,780.48 Da is present in control plasma but not in ovarian cancer plasma, and in (B), a protein peak of *Mr* 11,537.7 Da is present in ovarian cancer plasma but not in control plasma.

Wizard in the Ciphergen ProteinChip software 5.0. Peak intensities were then transferred to Biomarker Pattern's software to establish a decision tree that could most correctly classify the samples. Statistically significant peaks within the Mr range 2,000–30,000 Da were used to generate the classification model. Figure 2 shows the tree structure of the classification model using the univariate split selection strategy and sample distributions in analysis of SAX2 chip protein profiles. Three peaks, with Mr of 8,780.48, 11,537.7 and 5,147.06 Da, were the best determinants of the decision tree. At Node 1 (defined by peak 8,780.48), 25 controls and one cancer patient with peak intensities higher than 18.986 were classified into Terminal Node 1. The other 39 samples were further classified at Node 2 (defined by peak 11,537.7), where eight controls and two cancer patients with peak intensity of 0.626 or less were classified to Terminal Node 2. The other 29 samples entered

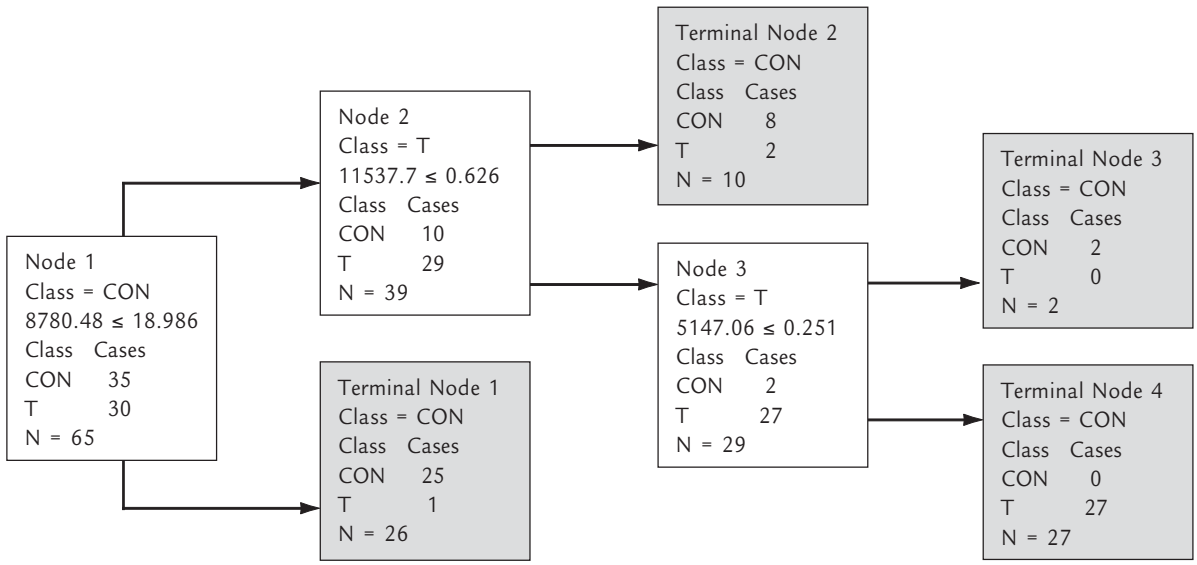

Figure 2. The decision tree and the distribution of samples in strong anion exchange-2 chip/surface-enhanced laser desorption/ionization (SELDI) analysis by univariate split. A tree structure of the classification model was generated to best classify the tumor (T) and control (CON) samples. In the open rectangles, the presence or absence of three SELDI-time-of-flight mass spectrometry peaks, with Mr of 8,780.48, 11,537.7, and 5,147.06 Da (with thresholds shown following the Mr), serves as root nodes to classify samples in a hierarchical way. Samples were classified into a lower level root node until the most correct classification was achieved in the terminal nodes (gray rectangles).

Node 3 (defined by peak 5,174.06), where two controls with peak intensities of 0.251 or less went to Terminal Node 3. The remaining 27 samples, which were all from cancer patients, were classified to Terminal Node 4. Overall, from the 65 samples, the model correctly classified all 35 controls and 27 of the 30 cancers, reaching a sensitivity of 90% and specificity of 100% in the learning set. Because there was no independent test sample, the cross-validation method was used to evaluate the decision tree, which gave a sensitivity of 84% and specificity of 89%.

The second split selection method was the linear combination split option for ordered predictor variables. Software analysis generated a linear combination equation:

–0.0944 (Mr 8,230.48) + 0.0901 (Mr 8,780.48) – 0.991 (Mr 11,537.7) = –0.177

where Mr 8,230.48, 8,780.482, and 11,537.7 Da are the best determinants. Figure 3 shows the decision tree distributions generated from the linear combination method. Among the 65 samples from which SELDI-TOF spectra were successfully generated, the model was able to discriminate 33 of the 35 controls and 29 of the 30 cancers, and reached a sensitivity of 97% and specificity of 94%. Cross validation of this classification tree gave a sensitivity of 84% and specificity of 89%.

Discussion

There is broad agreement that analysis of the human serum proteome has great potential for diagnosis and early detection of human disease. The challenges are immense given the complexity of the human proteome and the broad dynamic range and abundance of individual proteins. The key to unlocking this potential is the development of reproducible, sensitive, and specific technology for proteomic analysis. Recent advances in technology suggest that this may now be feasible. TOF MS technology offers a powerful and sensitive tool to study protein profiles in serum or plasma obtained from cancer patients and normal subjects. More recently, there has been considerable interest in analyzing the SELDI-MS spectral "proteomic pattern" [12–30]. The high dimensional array, created by the spectrum of thousands of peptides and their intensities, provides discriminatory power for separating any given set of case and control specimens. Although the limitations of SELDI-MS study design and its analysis have been discussed in some detail in the literature [16], the potential implications of such proteomic spectrum analysis for the identification of novel tumor markers is huge. It is possible that, in the future, the early detection of cancers will involve high-throughput proteomic profiling either alone or in combination with markers already in use. Validation of the methodology requires demonstration that the discriminatory algorithm is

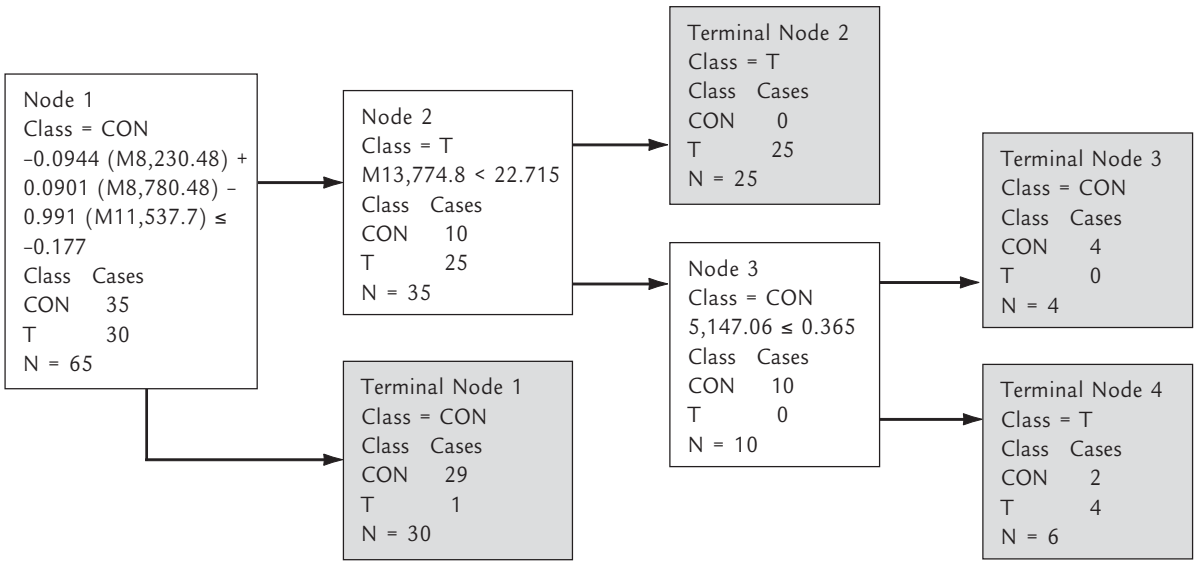

Figure 3. The decision tree and the distribution of samples in strong anion exchange-2 chip/surface-enhanced laser desorption/ionization (SELDI) analysis by linear combination split. A tree structure of the classification model was generated to best classify the tumor (T) and control (CON) samples. In the open rectangles, the presence or absence of five SELDI-time-of-flight mass spectrometry peaks, with Mr of 8,230.48, 8,780.482, 11,537.7, 13,774.8 and 5,147.065 Da (with thresholds shown following the Mr), serves as root nodes to classify samples in a hierarchical way. Samples were classified into a lower level root node until the most correct classification was achieved in the terminal nodes (gray rectangles).

reproducible among different laboratories and different sets of case–control specimens. This study demonstrates the feasibility of detecting protein signatures from cancer plasma using SELDI-MS and artificial intelligence. Although additional study is necessary to validate these patterns as unique diagnostic tools, these proteins are potentially useful in the diagnosis or monitoring of ovarian cancer, and, in an easier format, for antibody-based chip SELDI-TOF technology.

Using protein-chip technology, many studies have isolated several distinctive protein peaks in ovarian cancer serum/plasma. One of these has been further characterized and determined to be haptoglobin-α1 [7]. In our data, one peak (Mr 11,537.7 Da) was upreguated in cancer patients. This peak might be haptoglobin-α1 and, therefore, potentially a useful biomarker in the detection and monitoring of ovarian cancer. In order to get a suitable classification model to discriminate normal and tumor protein profiles, many statistical methods were applied to test the SELDI data profile. Appropriate baseline correction and peak intensity normalization is very important for SELDI data analysis [15,16]. In comparison with other reports [7, 12,31,32], these data showed high reproducibility, specificity, and sensitivity.

Recently, important concerns were raised on the validity of serum proteomic pattern analysis by mass spectrometry for early cancer diagnosis. First, there are uncertainties about whether or not it will be possible to identify subtle changes caused by early ovarian cancer in a low-abundance protein or protein fragment. Diamandis argues that this approach would identify high-abundance proteins in the circulation that are not released by the tumor, likely representing nonspecific epiphenomena of cancer [16]. If so, it may prove difficult to achieve adequate sensitivity for early detection of preclinical ovarian cancer using current technology. Second, there are many potential variables and consequently possible sources of bias related to differences between cases and controls, as well as variations in sample collection, processing, and storage. For example, in the Petricoin et al report, there was a 10-year difference in the mean age of the control group and the ovarian cancer group, so the differences observed could have been related to factors such as age or menopausal status [12]. Several of these points highlight the need for careful selection of samples in future proteomic studies. It is essential that studies utilize samples from carefully matched populations collected and stored under the same conditions.

In conclusion, up to now, SELDI-TOF MS is not sensitive enough to identify molecules in the subnanogram per milliliter range. It remains to be seen whether further refinements, such as fractionation enrichment techniques, will yield clinically useful diagnostic methods for cancer. Therefore, it seems desirable to know the identity of the biomarkers in the pattern in order to understand their significance in disease pathogenesis, as has been reported with the use of SELDI-MS to identify amyloid-β-peptide as a diagnostic marker for Alzheimer's disease [33–35] and α-defensin 1, 2, 3 as a favorable prognostic marker in acquired immune deficiency and contributor to anti-HIV-1 activity [36]. We hope in the future to identify these protein peaks and combine them with other markers such as CA125 or CA15-3 to assess therapeutic response and increase early detection of relapse of ovarian cancer.

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